

**Determining the Yeast Count in Maple Sirup
and a Collaborative Study of the Methods
and Sampling Techniques**

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Techniques for sampling and a method for determining the yeast count in maple sirup have been studied collaboratively in 22 laboratories. Statistical analysis of collaborators' results indicated that variation in shipping conditions had an adverse effect on the sirup samples and that difficulties in

preparing homogeneous cell suspensions in the sirup prior to subsampling caused wide variation in counts. Studies will continue.

Maple sirup because of its high sugar content (65.5° Brix) has enough osmotic pressure to inhibit the growth of most micro-

organisms. However, it is well-recognized by the industry and reported in the literature (1) that considerable losses of sirup occur because of the growth of microorganisms. In making maple sirup, sap is heated at its atmospheric boiling point, 212–219°F, for 1.5 to 2 hours. This heating period is usually sufficient to yield sterile sirup when it is drawn from the evaporator. However, bacterial, yeast, and mold spores are known to survive. Sirup is often subject to microbial contamination in later treatment and storage; spoilage occurs when conditions favorable to microbial growth are established.

Osmophilic and facultative osmophilic yeasts and molds are the most important spoilage organisms in maple sirup. Molds grow only on the sirup surface and then only when conditions conducive to spore germination exist, whereas the osmophilic yeasts grow throughout the sirup and produce a pungent odor accompanied by a pronounced "off" flavor. Excessive yeast growth also causes turbidity and gas production. When growth occurs in sealed containers, gas causes explosive rupture of the container. In open-top bulk holding tanks, yeast contaminations first appear at the sirup surface and extend deeper into the sirup as fermentation progresses. Yeasts isolated from contaminated sirups have exhibited good growth on wort agar and on acidified potato dextrose agar, which indicates that they are facultative organisms.

A method for the quantitative evaluation of viable yeasts in maple sirup has never been reported in the literature. Such a method would permit measuring the yeast population at low levels in the sirup before or during bulk storage and would provide the means for evaluating the corrective action taken to control subsequent yeast growth and spoilage of the sirup.

A method is described for determining the number of yeasts in maple sirup, including sampling techniques; results of a collaborative study of the method are also presented.

METHOD

Apparatus¹ and Reagents

- (a) *Hypodermic syringe*.—5 ml (Luer Lok).
- (b) *Hypodermic needle*.—18 or 20 gauge (Luer Lok).
- (c) *Sample bottle*.—Wide mouth, screw neck, 4 oz, molded plastic cap with tin foil liner.
- (d) *Spatula*.— $\frac{1}{2} \times 4$ " blade.
- (e) *Dilution bottles*.—160 ml, $45 \times 45 \times 140$ mm.
- (f) *Pipets*.—Serological, TD 1.0 ml with 0.1 ml graduations.
- (g) *Petri dishes*.— 100×15 mm.
- (h) *Wort agar culture medium*.—Boil 15.00 g malt extract, 0.78 g peptone, 2.75 g dextrin, 2.35 g glycerol, 1.00 g dipotassium phosphate, 1.00 g ammonium chloride, 12.75 g maltose, and 20.00 g agar until dissolved. Sterilize by autoclaving at 15 lb pressure for 15 min.
- (i) *Phosphate buffer stock solution*.—0.25M. Dissolve 34.0 g KH_2PO_4 in 500 ml H_2O , adjust to pH 7.2 with 1N NaOH, and dilute to 1 L.
- (j) *Phosphate buffer dilution water*.—Add 1.25 ml 0.25M phosphate buffer stock solution (i) to 1 L H_2O . Dispense in dilution bottles and sterilize by autoclaving at 15 lb pressure for 20 min.

Procedure

Invert wide-mouth bottle containing laboratory sirup sample 10 times to disperse any bottom sediment; invert slowly to prevent air bubbles. Remove cap and stir sirup slowly with sterile $\frac{1}{2}$ " blade spatula to obtain uniform suspension without air bubbles. If gas bubbles form, let sample stand until bubbles disappear.

Insert tip of 5 ml syringe, without needle attached, 1" below surface of sirup. Draw 5.5–6.0 ml sample into syringe. Wipe excess sirup from syringe barrel with 4×4 " sterile gauze pad wetted with 95% ethyl alcohol. Hold syringe in upright position and attach 18 or 20 gauge needle. Hold needle-syringe assembly with sterile gauze pad and expel excess sirup and air bubbles from syringe and needle by bringing plunger exactly to 5 ml graduation.

Make 10^{-1} dilution of sirup by expelling 5 ml of sirup completely from syringe into 45 ml sterile phosphate buffer dilution blank. Shake inoculated dilution blank vigorously for

¹ This report of the Associate Referee was presented at the Eightieth Annual Meeting of the Association of Official Analytical Chemists, Oct. 10–13, 1966, at Washington, D.C.

¹ Apparatus a, b, c, d, f, and g are sterilized in a hot air oven at 160°C for 1 hr except when items f and g are obtained as "single use" sterile plastic.

10 sec, transfer 1.0 ml to petri dish by using 1.0 ml pipet for 10^{-1} dilution plate, and transfer 0.1 ml of 10^{-1} dilution with 1 ml pipet to petri dish for 10^{-2} dilution plate. Transfer 1.0 ml of 10^{-1} dilution to 99 ml sterile dilution blank for 10^{-3} dilution. Prepare 10^{-3} and 10^{-4} dilution plates by using 1.0 and 0.1 ml volumes of 10^{-3} sirup dilution, respectively. Transfer 1.0 ml of 10^{-3} dilution to 99 ml sterile dilution blank to make 10^{-5} sirup dilution. Prepare 10^{-5} and 10^{-6} dilution plates by using 1.0 and 0.1 ml volumes 10^{-5} sirup dilution, respectively. Pour 10-12 ml liquefied wort agar at 42-44°C into each plate and mix with diluted culture. After agar has solidified, invert plates and incubate for 5 days between 21 and 25°C. Count plates on fifth day on a Quebec Colony Counter or its equivalent.

Results and Discussion

This method and sampling technique were tested for precision and reliability before the collaborative test by the techniques recommended by Youden (2). Results of these tests on 10 replicated analyses were concordant: standard deviation(s) of 9.9×10^4 from a mean cell count of 1.67×10^6 and a coefficient of variation of 5.9%.

Viscous maple sirup can be accurately subsampled for yeast counts by weighing. However, it is difficult to obtain a specific amount of sample, and the method does not permit repetitive replicated samples. Before collaborative study of this method, a subsampling procedure was developed to permit replicated samples and minimize variation in sample size. Hypodermic syringes were compared with pipets as to accuracy and precision for obtaining repetitive and replicate samples. The weights of maple sirup delivered by 5.0 and 10.0 ml hypodermic syringes and by 5.0 and 10.0 ml TD pipets are given in Table 1. Data show that the syringes delivered precise and concordant weights; standard deviations were low, i.e., 0.071 and 0.021 for the 5.0 and 10.0 ml syringes, respectively. Table 1 also shows that the desired sample replication cannot be obtained with a pipet; standard deviations for the weighed volumes delivered by the pipets were 0.145 for the 5.0 ml pipets and 0.062 for the 10.0 ml pipets. The precision and accuracy of the replicated volumes

Table 1. Comparison of precision of syringes vs pipets in delivery of maple sirup by wt (g)^a

Aliquot No.	5 ml Syringe	5 ml Pipet	10 ml Syringe	10 ml Pipet
1	6.41	5.91	12.71	12.29
2	6.23	5.96	12.67	12.43
3	6.39	6.00	12.72	12.24
4	6.37	6.13	12.72	12.23
5	6.37	5.73	12.69	12.41
Mean	6.35	5.94	12.70	12.32
Std dev.	0.071	0.145	0.021	0.062

^a Specific gravity of sirup, 1.32.

of maple sirup obtained by a calibrated hypodermic syringe with needle met the requirements for this study.

Collaborative Study

To evaluate the method and sampling technique under a variety of conditions, a collaborative program was designed with different laboratories and two samples of sirup, one with a high yeast count and the other with a low yeast count at the time of shipment. Two lots of sirup were inoculated with a facultative osmophilic *saccharomyces* yeast that had been isolated from a naturally contaminated maple sirup. This organism grows very slowly; it usually requires 3 week incubation before actively producing gas in its fermentation of maple sirup. The sirup sample with a high yeast count was inoculated 6 weeks before subsampling and shipment, at which time it contained 7×10^5 yeast cells per ml and was designated as Sample A. The other sample, inoculated and subsampled on the day of shipment, contained 2×10^3 yeast cells per ml and was designated Sample B. All samples were sent to the collaborators on the same day.

Twenty-four microbiologists participated in the 1966 collaborative study. Each analyst was requested to analyze only one of the two samples, i.e., 12 collaborators were sent Sample A and the other 12 were sent Sample B. Collaborators were requested to make a minimum of five separate determinations including sampling, plating, and counting.

All samples were sent via first class parcel post with instructions for special handling. Air mail was not used because subfreezing temperatures would have been encountered

in shipment. Samples sent to collaborators in the East were in transit only 24 hours; samples sent to midwestern collaborators were in transit 5 days. To compensate for the varying times that the samples were in transit, each collaborator was requested to hold his sample at room temperature until a given date, 10 days after shipment, to provide adequate time for all deliveries and to assure that all samples would be held under the same conditions and that the cultures would be exactly the same age at the time of study.

Results obtained by the collaborators are given in Tables 2 and 3. Data from 11 collaborators are tabulated for Samples A and B, respectively; one sample in each group was lost in transit.

The interlaboratory results for Sample A (Table 2) showed considerable variation as indicated by the mean (\bar{x}) of the collaborators' yeast counts. The coefficient of variation evaluates intralaboratory standard deviations (S) on a comparable basis; the data show definite grouping. Two collaborators had coefficients of variation of 3.3 and 3.57%, respectively. Five other collaborators had coefficients of variation ranging from 11.2 to 15.2%. The coefficients of variation for the remaining collaborators ranged from 19.1 to 115.6%.

The 1966 collaborators' yeast counts for Sample B are given in Table 3. The means

of the individual collaborators' yeast counts (\bar{x}) showed a similar wide interlaboratory variation as that in Table 2. Intralaboratory results, as indicated by the coefficient of variation, also showed a grouping of results. The coefficient of variation for 6 collaborators ranged from 6.3 to 11.9%. Results of three collaborators had coefficients of variation ranging from 20.3 to 39.3%, and the remaining collaborators had coefficients of variation of 78.2 and 79.1%, respectively.

Data for the two sets of samples indicate that this counting and sampling procedure was successfully used by 50% of the collaborators and that the method needs to be improved by further study. It is apparent that not all collaborators were able to prepare homogeneous suspensions of the yeast cells. Another source of error, perhaps the major one, is the different conditions of incubation of the samples while in transit.

No collaborators raised any serious questions, although one collaborator felt that the syringe sampling technique was too cumbersome for use on an industrial scale. Another collaborator noted that small mixers capable of mixing viscous liquids without incorporating air bubbles have become commercially available; he suggested that one of these might be adapted for aseptic mixing of contaminated sirups to prepare homogeneous mixtures for subsampling. This suggested technique will be given further study.

Table 2. Summary of collaborative yeast counts for maple sirup Sample A^a; 5 determinations per collaborator

Coll.	Range, cells/ml $\times 10^{-3}$		$\bar{x} \times 10^{-3}$	$S \times 10^{-3}$	$V, \%$
	Low	High			
1	1	84	33.2	38.4	115.6
2	270	290	280	10	3.57
3	260	1100	680	376.5	55.3
4	550	810	682	130.6	19.1
5	710	770	754	25.1	3.3
6	900	1200	1080	145	13.4
7	1000	2100	1540	439	28.5
8	1400	2000	1640	250	15.2
9	1500	2100	1720	238.7	13.8
10	1500	1900	1740	167	11.2
11	1800	2300	2020	228	11.3

^a \bar{x} = mean of determinations; S = std dev. of determinations; V = coefficient of variation.

Table 3. Summary of collaborative yeast counts for maple sirup Sample B^a; 5 determinations per collaborator

Coll.	Range, cells/ml $\times 10^{-3}$		$\bar{x} \times 10^{-3}$	$S \times 10^{-3}$	$V, \%$
	Low	High			
12	5	8	6.4	1.3	20.3
13	2	20	8.6	6.8	79.1
14	8	21	13.2	5.2	39.3
15	14	17	16	1.2	7.5
16	22	28	25	2.9	11.6
17	5	80	44	35.2	78.2
18 ^b	27	88	60.1	20.6	34.2
19	140	190	174	20.7	11.9
20	183	210	195	11.8	6.3
21	290	320	296	23	7.7
22	210	490	336	39.4	11.7

^a See footnote a, Table 2.

^b 10 determinations.

Recommendation

It is recommended that further studies be conducted on methods for counting yeasts in maple sirup with emphasis on the preparation of homogeneous suspensions of cells in sirup and on methods that will compensate for different incubation conditions of the sample while it is in transit.

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